The role of platelet adhesion receptor GPIbα far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis

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GPIbα binding to von Willebrand factor (VWF) exposed at a site of vascular injury is thought to be the first step in the formation of a hemostatic plug. However, our previous studies in VWF-deficient mice demonstrated delayed but not absent arterial thrombus formation, suggesting that, under these conditions, GPIbα may bind other ligands or that a receptor other than GPIbα can mediate platelet adhesion. Here, we studied thrombus formation in transgenic mice expressing GPIbα in which the extracellular domain was replaced by that of the human IL-4 receptor (IL4Rα/GPIbα-tg mice). Platelet adhesion to ferric chloride-treated mesenteric arterioles in IL4Rα/GPIbα-tg mice was virtually absent in contrast to avid adhesion in WT mice. As a consequence, arterial thrombus formation was inhibited completely in the mutant mice. Our studies further show that, when infused into WT recipient mice, IL4Rα/GPIbα-tg platelets or WT platelets lacking the 45-kDa N-terminal domain of GPIbα failed to incorporate into growing arterial thrombi, even if the platelets were activated before infusion. Surprisingly, platelets lacking β3 integrins, which are unable to form thrombi on their own, incorporated efficiently into WT thrombi. Our studies provide *in vivo* evidence that GPIbα absolutely is required for recruitment of platelets to both exposed subendothelium and thrombi under arterial flow conditions. Thus, GPIbα contributes to arterial thrombosis by important adhesion mechanisms independent of the binding to VWF.

platelets | adhesion

Platelet adhesion to the extracellular matrix (ECM) is a key step in thrombus formation as found in hemostasis and thrombosis. The interaction of platelet receptor GPIbα and von Willebrand factor (VWF) exposed in the ECM is commonly accepted as the key event required for the initial tethering of platelets along the damaged vascular wall, especially under conditions of high shear stress (1–3). Most of the data supporting this model are derived from *in vitro* studies in flow chamber models, where whole blood or isolated platelets are perfused under physiological flow conditions over adhesive surfaces such as collagen or VWF. Using mice deficient in VWF (4), we previously evaluated the role of VWF in ferric chloride-induced thrombosis in mesenteric arterioles (5). Compared with controls, the number of VWF$^{-/-}$ platelets tethering along the injured vessel wall was initially reduced by ≈80%, causing a delay of several minutes in thrombus formation. However, VWF$^{-/-}$ mice managed to form stable thrombi, which in ≈50% of cases led to complete occlusion of the injured arteriole (5). In many mice, a high-shear channel formed that persisted for >25 min. The presence of such channels also was reported for hemostatic plugs in pigs with von Willebrand's disease (6). These data suggested that a ligand for GPIbα other than VWF was expressed in the vessel wall, or that a receptor other than GPIbα-mediated platelet adhesion in injured arterioles of VWF$^{-/-}$ mice.

The GPIb-V-IX receptor complex consists of four gene products, GPIbα, GPIbβ, GPIX, and GPV (2, 7, 8). Approximately 25,000 copies of GPIb-IX and 12,000 copies of GPV are expressed on resting platelets. The ligand-binding site for VWF is located within the 45-kDa N-terminal region of GPIbα. Recent studies demonstrated that the GPIb-V-IX complex plays a broader role in vascular biology, because it also mediates the interaction of resting platelets with activated leukocytes through its binding to Mac-1 (9, 10) and activated endothelial cells through its binding to P-selectin (11). Furthermore, GPIbα interacts with thrombin (12, 13), high molecular weight kininogen (14), coagulation factors XI and XII (15, 16), and TSP-1 (17). Upon ligand binding, the GPIb-V-IX receptor complex can induce signals leading to calcium mobilization, the rearrangement of the cytoskeleton, granule release, and activation of αIIbβ3 integrin (18, 19).

The importance of the GPIb-V-IX receptor for hemostasis is shown by the strong bleeding diathesis found in Bernard–Soulier syndrome (BSS) patients lacking the GPIb-V-IX complex on the platelet surface or in patients lacking VWF (1, 20). A similar bleeding phenotype was reported for mice deficient in GPIbα (21). However, additional characteristics of BSS, such as marked thrombocytopenia and giant platelets, are also found in GPIb$\alpha^{-/-}$ mice, making it difficult to specifically address the role of GPIbα in thrombosis.

Therefore, we decided to evaluate thrombosis in mice expressing GPIbα, in which most of the extracellular domain has been replaced by the α-subunit of the human IL-4 receptor (IL4Rα) (22). Mice transgenic for this chimeric receptor (IL4Rα/GPIbα-tg) on the GPIb$\alpha^{-/-}$ background are characterized by normal platelet size and a platelet count of ≈65–70% of WT mice. Like complete GPIbα deficiency, lack of the extracellular domain of GPIbα results in a severe bleeding phenotype (22).

We demonstrate here that platelet adhesion to the exposed extracellular matrix is completely inhibited in arterioles of IL4Rα/GPIbα-transgenic mice. The comparison with the milder phenotype of VWF$^{-/-}$ animals strongly suggests the existence of a ligand other than VWF for GPIbα in the injured vessel. Furthermore, our studies show that surface expression of GPIbα, but not β3 integrin, on circulating platelets is crucial for their recruitment into arterial thrombi.

Results

***In Vitro* Characterization of IL4Rα/GPIbα-tg Platelets.** We first wanted to rule out possible compensatory effects of this genetic

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Abbreviations: ECM, extracellular matrix; RT, room temperature; VWF, von Willebrand factor.

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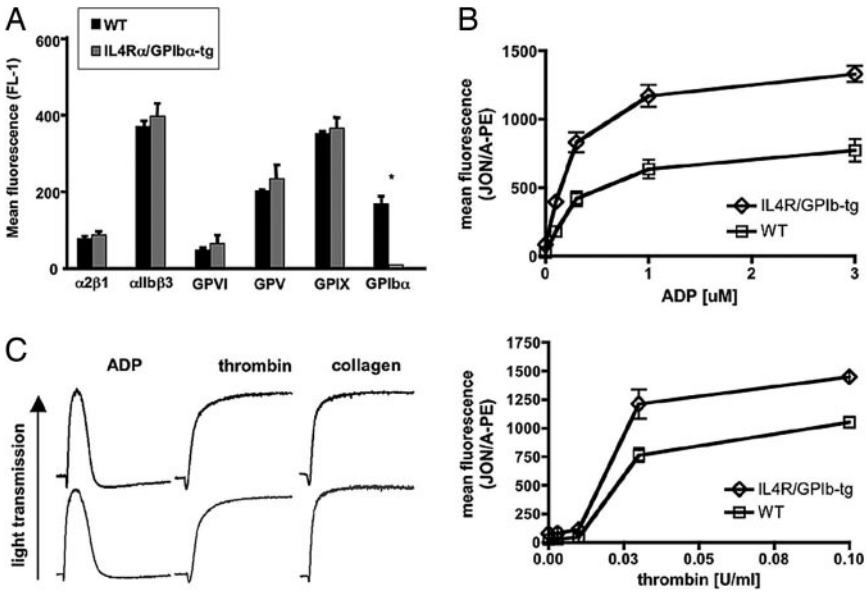

Fig. 1. *In vitro* characterization of IL4Rα/GPIbα-tg platelets. (*A*) IL4Rα/GPIbα-tg platelets show normal surface expression of important adhesion receptors. WT or IL4Rα/GPIbα-tg platelets were stained with FITC-labeled antibodies against the indicated adhesion receptors and immediately were analyzed by flow cytometry. $n = 4$; *, $P < 0.0001$. (*B*) αIIbβ3 activation. Washed WT (squares) or IL4Rα/GPIbα-tg (diamonds) platelets were stimulated with the indicated concentrations of thrombin or ADP (in the presence of 5 μM concentration of the thromboxane A_2 mimetic U46619) for 5 min, stained with an antibody recognizing activated αIIbβ3 (JON/A-PE), and immediately analyzed by flow cytometry. $n = 6$. (*C*) Standard aggregometry traces of WT (black line) or IL4Rα/GPIbα-tg (gray line) platelets stimulated with 1 μM ADP, 10 μg/ml collagen, or thrombin (0.04 units/ml). Platelet aggregation (light transmission) was monitored over 12 min. Results are representative of three experiments. No defects in receptor expression and αIIbβ3 activation were observed in IL4Rα/GPIbα-tg platelets.

modification, which could result in altered surface expression of important platelet adhesion receptors. As shown in Fig. 1*A*, no significant differences could be observed between WT and IL4Rα/GPIbα-tg platelets with regard to the surface expression of integrins α2β1 and αIIbβ3, or GPVI. Staining for both GPV and GPIX, members of the Ib-V-IX complex, also showed no significant difference between WT and IL4Rα/GPIbα-tg platelets, whereas staining for GPIbα was observed on WT but not IL4Rα/GPIbα-tg platelets.

In addition to the expression level of surface adhesion receptors, we studied αIIbβ3 integrin activation and calcium flux in WT and IL4Rα/GPIbα-tg platelets stimulated with various agonists. These studies are important because they provide information on the function of surface-expressed agonist receptors, intracellular signaling pathways, and the major platelet integrin responsible for platelet adhesion and aggregation. No significant shift in the dose–response to ADP (in the presence of 5 μM concentration of the thromboxane A2 mimetic U46619) or thrombin was observed in flow cytometry studies aimed to directly measure αIIbβ3 activation. Interestingly, at all agonist concentrations tested, binding of probes detecting the activated form of αIIbβ3 such as the monoclonal antibody JON/A-PE (Fig. 1*B*) or antibodies detecting surface-bound fibrinogen (data not shown) was significantly stronger on IL4Rα/GPIbα-tg platelets when compared with WT platelets. In addition, no significant differences in calcium flux were observed between WT and mutant platelets stimulated with ADP, U46619, or thrombin (data not shown). The ability of αIIbβ3 to bind ligands and mediate platelet aggregation was tested further in standard aggregometry, where no differences between WT and IL4Rα/GPIbα-tg platelets were found (Fig. 1*C*).

Impaired Arterial Thrombosis in IL4Rα/GPIbα-tg Mice. To visualize the adhesive function of IL4Rα/GPIbα-tg platelets *in vivo*, we performed intravital microscopy studies in a model of arterial thrombosis. In this model, platelets from a donor mouse are isolated, washed, labeled with calcein-AM, and infused into a recipient mouse of the same genetic background. Shortly after visualization of a mesenteric arteriole, the vessel wall was injured by application of ferric chloride ($FeCl_3$), and the adhesion of labeled platelets and the formation of thrombi at the site of injury was observed. The numbers of fluorescently labeled platelets deposited on vessel walls between 3–5 min after injury were counted over a 400-μm length of vessel. Whereas strong tethering of platelets to the injured vessel wall was observed in WT mice 3–5 min after application of $FeCl_3$ (192.0 ± 17.6 platelets per min), tethering of platelets was virtually absent (3.1 ± 1.1 platelets per min) in IL4Rα/GPIbα-tg mice ($P < 0.0001$). Consequently, thrombus formation in arterioles of IL4Rα/GPIbα-tg mice was completely absent throughout the 40-min course of the experiment, whereas vessel occlusion in WT mice was reached 15.9 ± 2 min after application of $FeCl_3$ (Fig. 2). Thus, GPIbα plays an essential role in the initial platelet adhesion that leads to arterial thrombosis.

IL4Rα/GPIbα-tg Platelets or Platelets Missing the 45 kDa N-Terminal Region of GPIbα Do Not Incorporate into Growing Arteriolar Thrombi in WT Mice, Even When Preactivated *in Vitro*. In this model, we propose to study which surface receptors are needed for platelet adhesion to a thrombus whose formation is driven by WT platelets. To test whether IL4Rα/GPIbα-tg platelets also are defective in their adhesion to an already existing thrombus, we infused WT mice with WT platelets labeled with calcein-green together with IL4Rα/GPIbα-tg platelets labeled with calcein-red/orange (or vice versa) to reach a total of 2% labeled platelets for both genotypes. Whereas labeled WT platelets showed normal adhesion to the damaged vascular wall and strong incorporation into the growing thrombus, the adhesion of labeled IL4Rα/GPIbα-tg platelets to the ECM and to the growing thrombi was strongly impaired (Fig. 3). To compare the incorporation into a growing thrombus of WT and IL4Rα/GPIbα-tg platelets, two independent observers assigned a score from 0 to

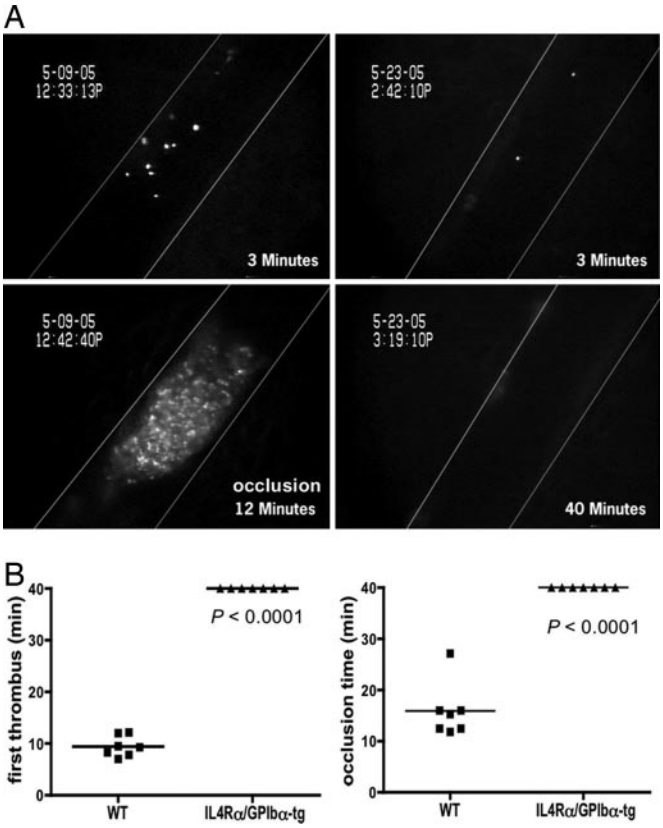

Fig. 2. Platelet adhesion and thrombus formation in $FeCl_3$-injured arterioles. (*A*) WT (*Left*) or IL4Rα/GPIbα-tg (*Right*) mice were injected with calcein-green-labeled platelets of the respective genotype, and platelet adhesion was monitored in mesenteric arterioles upon application of $FeCl_3$. Images show platelet adhesion and thrombus formation in arterioles at the indicated times after application of $FeCl_3$. The direction of blood flow is from upper right to lower left. Note that adhesion in arterioles from IL4Rα/GPIbα-tg mice is virtually absent for the entire observation period (40 min). (*B*) Comparison of the time of the appearance of the first thrombus (>20 μm) and the time of occlusion in $FeCl_3$-treated mesenteric arterioles of WT and IL4Rα/GPIbα-tg mice. No thrombi formed in the mutant mice during the 40 min of observation. Movies showing platelet adhesion/thrombus formation in injured arterioles in real time can be found in supporting information.

10 for IL4Rα/GPIbα-tg platelets, with "0" representing "no incorporation" and "10" representing "incorporation efficiency of WT platelets." IL4Rα/GPIbα-tg platelets showed significantly less incorporation than WT platelets (score = 0.9 $\pm$ 0.5; $P = 0.001$; $n = 5$).

A similar defect was observed when we studied the adhesive function of WT platelets lacking the 45-kDa N-terminal region of GPIbα (WT-45-kDa platelets), generated by treatment with *O*-sialoglycoprotein endopeptidase for 30 min *in vitro* (23). The efficiency of GPIbα cleavage was verified by flow cytometry with antibodies directed against epitopes located within and outside the 45-kDa N-terminal domain of GPIbα (Fig. 4*A*). Similar to IL4Rα/GPIbα-tg platelets, tethering of WT-45-kDa platelets was almost completely absent (4.0 $\pm$ 2.8 platelets per min compared with 178 $\pm$ 23 platelets per min for WT; $P < 0.002$), and so was their incorporation into a growing WT thrombus (Fig. 4*B*). Together, these data indicate that platelets lacking the N-terminal ligand-binding domain on GPIbα are incapable of incorporating into a growing WT thrombus under arterial flow conditions.

To rule out that impaired platelet activation in response to thrombin or other agonists is responsible for the adhesion defect

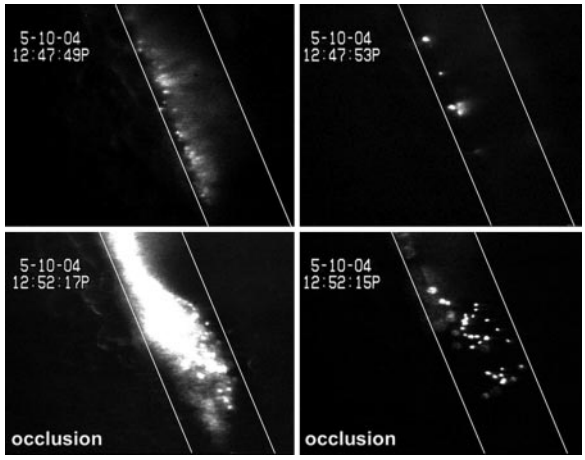

Fig. 3. IL4Rα/GPIbα-tg platelets show strongly impaired incorporation into a growing WT thrombus. Washed platelets were labeled with calcein-green (WT; *Left*) or calcein-red/orange (IL4Rα/GPIbα-tg; *Right*) and transfused into the same WT recipient mouse to reach a total of 2% labeled platelets for each genotype. Vascular injury was induced by application of $FeCl_3$, and thrombus growth was monitored until blood flow stopped (occlusion). Although WT platelets incorporated avidly into the thrombus, only a few entrapped IL4Rα/GPIbα-tg platelets could be seen. Results are representative of five independent experiments.

of IL4Rα/GPIbα-tg platelets, we studied the incorporation of thrombin-activated platelets into WT thrombi. It was shown that thrombin-activated platelets are not cleared upon infusion into mice (24, 25). Platelet activation was confirmed by examining P-selectin expression before infusion (data not shown). The preactivation of IL4Rα/GPIbα-tg platelets did not stimulate their adhesion to either ECM or thrombi (Fig. 4*C*).

β3 Integrin-Deficient Platelets Incorporate into Growing WT Thrombi in a GPIbα-Dependent Manner. Because platelet recruitment to thrombi strongly depended on the expression of GPIbα, we wondered whether platelets lacking integrin αIIbβ3, the key receptor mediating platelet aggregation (26), are capable of incorporating into a growing WT thrombus. To our surprise, we observed effective incorporation of $\beta3^{-/-}$ platelets into WT thrombi, both in the early phase of thrombus growth and at the time of occlusion (Fig. 5*A*). In contrast to IL4Rα/GPIbα-tg platelets, the incorporation of $\beta3^{-/-}$ platelets was not significantly different from that of WT platelets (incorporation score = 9.25 $\pm$ 0.75; $P = 0.5$). However, treatment with *O*-sialoglycoprotein endopeptidase to remove the 45-kDa N-terminal domain of GPIbα abolished the incorporation of $\beta3^{-/-}$ platelets (Fig. 5*B*), suggesting that GPIbα, but not αIIbβ3, is the main receptor facilitating platelet recruitment and adhesion to thrombi under arterial flow conditions.

Discussion

Here we show that the lack of the extracellular domain of GPIbα abolishes platelet adhesion to sites of vascular damage in injured arterioles (Fig. 2). This defect in platelet adhesion is the strongest that our group has observed so far by using this model in genetically modified mice. The phenotype is even more severe than that of mice deficient in integrin αIIbβ3, the major integrin mediating platelet aggregation, which show significant platelet tethering but fail to form arterial thrombi (27–29) (Richard O. Hynes, personal communication). In addition to their inability to adhere to the exposed ECM, IL4Rα/GPIbα-tg platelets or platelets lacking the 45-kDa N-terminal domain of GPIbα infused into WT mice were unable to incorporate into growing WT thrombi (Figs. 3 and 4). This finding is consistent with two

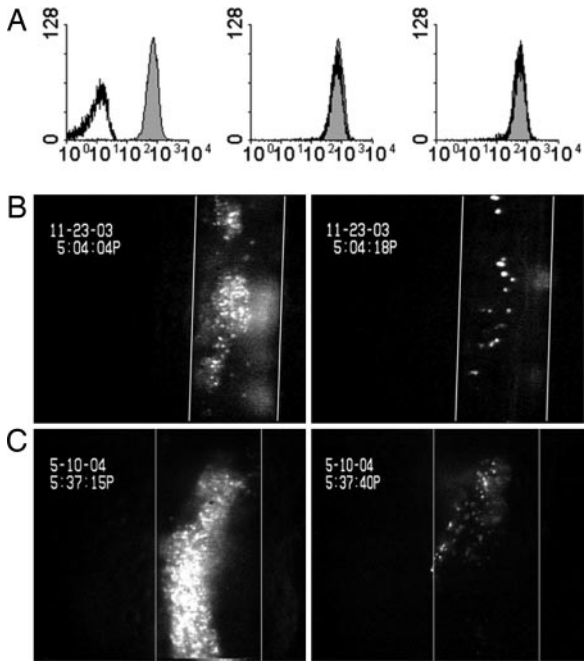

Fig. 4. Platelets lacking the 45-kDa N-terminal domain of GPIbα are unable to incorporate into a growing WT thrombus. (*A*) Washed platelets were treated with *O*-sialoglycoprotein endopeptidase. Removal of the 45 kDa N-terminal domain of GPIbα was monitored by flow cytometry with antibodies against epitopes within (p0p4; *Left*) and outside (p0p5; *Center*) this domain of the receptor (8). Antibodies against GPV (*Right*) were used as a control. (*B*) Platelets were labeled with calcein-green (WT; *Left*) or calcein-red/orange (WT lacking 45-kDa N-terminal domain; *Right*) and transfused into the same WT recipient mouse. Vascular injury was induced by application of $FeCl_3$ and thrombus growth was monitored. Results are representative of five independent experiments. (*C*) WT mice were infused with WT platelets labeled in calcein-green (*Left*), and thrombosis was induced by $FeCl_3$. When the first thrombus <10 μm in diameter was observed, thrombin-activated IL4Rα/GPIbα-tg platelets labeled with calcein-red/orange (*Right*) were infused into the same mouse. Thrombus growth then was monitored until blood flow stopped (occlusion). Only a few activated IL4Rα/GPIbα-tg platelets were entrapped in the occlusive thrombus.

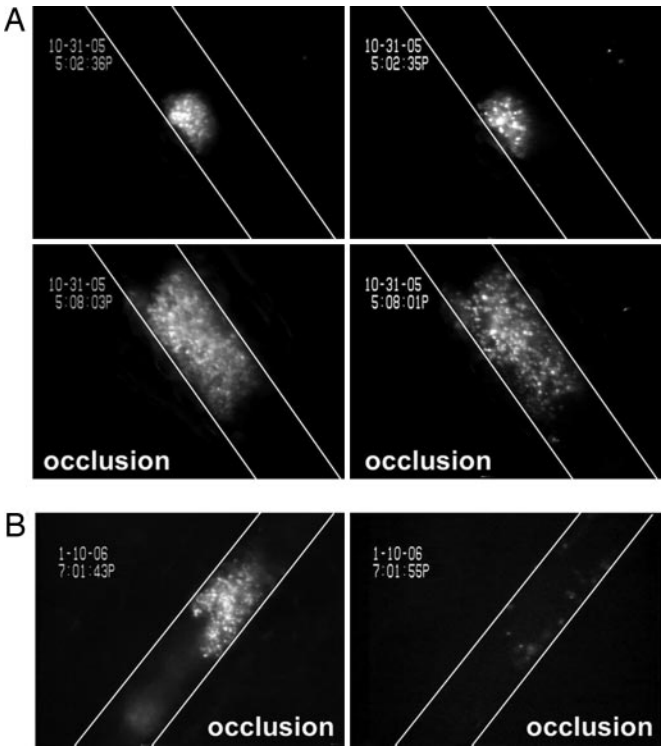

Fig. 5. Incorporation of β3-deficient platelets into a growing WT thrombus requires the extracellular domain of GPIbα. (*A*) Platelets were labeled with calcein-green (WT; *Left*) or calcein-red/orange ($\beta3^{-/-}$; *Right*) and transfused into the same WT recipient mouse. Vascular injury was induced by application of $FeCl_3$, and thrombus growth was monitored until blood flow stopped (occlusion). Results are representative of five independent experiments. (*B*) Arterial thrombosis was studied in a WT recipient mouse infused with $\beta3^{-/-}$ platelets (calcein-green; *Left*) and $\beta3^{-/-}$ platelets lacking the 45-kDa N-terminal domain of GPIbα (calcein-red/orange; *Right*). Only platelets containing the N-terminal domain of GPIbα can incorporate successfully into thrombi.

previous reports demonstrating *in vitro* that the GPIb–VWF interaction plays a key role in platelet thrombus growth, i.e., the adhesion of platelets to already adherent platelets (30, 31). However, our findings provide important insights into the mechanism(s) underlying platelet-platelet cohesion, because we were able to demonstrate that the incorporation of mutant platelets into a growing WT thrombus in arterioles does not require the major platelet integrin, αIIbβ3 (Fig. 5*A*). This finding is surprising given that the absence of functional αIIbβ3, either due to the addition of function-blocking compounds or genetic deficiency, leads to complete inhibition of thrombus formation under conditions of arterial flow (26–28, 32) (Richard O. Hynes, personal communication). We do not understand how β3 integrin-deficient platelets manage to firmly adhere to WT platelets, but we could demonstrate that their recruitment requires the extracellular domain of GPIbα (Fig. 5*B*). One possibility would be that GPIbα facilitates the tethering of $\beta3^{-/-}$ platelets, mediated by a GPIbα ligand such as VWF, which could cross-link the incoming platelet to another GPIbα or to an integrin, such as αIIbβ3 on the thrombus surface. Firm adhesion of newly recruited platelets then may be mediated by activated β1 integrins such as $\alpha5\beta1$ (fibronectin receptor). Fibronectins are present not only in the ECM but are also found in plasma and platelets (33). This idea might be supported by our recent findings showing that the lack of plasma fibronectin or even a reduction in its levels leads to significantly prolonged occlusion time in injured arterioles in mice because of the constant shedding of platelets from the forming thrombus (34, 35). Alternatively, an integrin-independent adhesion process could direct platelet incorporation into the WT thrombus, possibly entirely dependent on the interaction of GPIbα with one (or more) of its ligands. Interestingly, while this paper was under review, Ruggeri *et al.* (36) reported that platelet aggregation under very high arterial shear conditions *in vitro* requires GPIbα and VWF, but is independent of platelet activation (and, therefore, integrin αIIbβ3). Thus, there is growing evidence that GPIbα can mediate firm adhesion of nonactivated platelets independent of the engagement of αIIbβ3.

It has been demonstrated that integrin cytoplasmic domains expressed as transgenic chimeric proteins can behave as inhibitors of endogenous integrin activation. For example, their cytoplasmic domains can act as a "sink" for intracellular signaling molecules, which are limited in quantities (37–39). One could imagine that, similarly, the expression of the IL4Rα/GPIbα chimeric protein could negatively affect platelet signaling/function. However, this hypothesis provides an unlikely explanation for the adhesion defect observed in IL4Rα/GPIbα-tg platelets, because we can recapitulate the adhesion deficit in WT platelets by the enzymatic removal of the 45-kDa N-terminal domain of GPIbα (Fig. 4). Furthermore, IL4R/GPIbα-tg platelets showed normal calcium flux and αIIbβ3 activation when activated with various physiological platelet agonists (Fig. 1). In fact, we observed increased binding of probes detecting the

activated form of αIIbβ3 on stimulated IL4R/GPIbα-tg platelets (Fig. 1*B*). Most likely, the better access of such probes is explained by the absence of the heavily glycosylated GPIbα extracellular domain. In any case, we were unable to detect any defect in the activation of IL4Rα/GPIbα-tg platelets.

The best known ligand for GPIbα is VWF. Interestingly, the phenotype of mice lacking the extracellular domain of GPIbα is far more severe than that of mice deficient in VWF. Examined in the same model of arteriolar injury, the number of $VWF^{-/-}$ platelets tethering along the injured vessel wall is ≈20% of WT, whereas we observed <2% of WT in the IL4Rα/GPIbα-tg mice. The reduced but not absent platelet adhesion observed in $VWF^{-/-}$ mice causes a delay of several minutes in thrombus formation. However, unlike IL4Rα/GPIbα-tg mice, the $VWF^{-/-}$ mice manage to form stable thrombi, which in ≈50% of cases lead to complete occlusion of the injured arteriole (5). The strikingly stronger antithrombotic phenotype of IL4Rα/GPIbα-tg mice compared with $VWF^{-/-}$ mice is surprising for several reasons. First, VWF has been established in previous studies as the major ligand for GPIbα, at least with regard to platelet adhesion to a thrombogenic surface under conditions of high shear stress (3, 40). Second, VWF is also a prominent ligand for the major platelet integrin, αIIbβ3. The interaction of αIIbβ3 with VWF has been shown to be an important step in platelet adhesion and thrombus formation, both *in vitro* and *in vivo* (3, 41, 42). Finally, VWF is a carrier protein for factor VIII (FVIII), a plasma clotting factor important for the generation of thrombin at sites of vascular damage. In human and mouse type 1 von Willebrand disease, markedly reduced VWF levels lead to a decrease in the plasma concentration of FVIII as a result of the lack of protection against proteolysis provided by VWF (4, 43–45). Thus, one might have expected a similar or stronger antithrombotic phenotype in $VWF^{-/-}$ mice compared with mice lacking the extracellular domain of GPIbα.

The strong adhesion defect of IL4Rα/GPIbα-tg platelets observed in injured arterioles shows that (*i*) other receptors such as the collagen receptor GPVI (46) cannot support platelet adhesion in the absence of GPIbα, and (*ii*) a ligand(s) other than VWF can support GPIbα-dependent platelet adhesion. Thrombospondin-1 (TSP-1) is one likely candidate, because it is has been identified as an alternative ligand to VWF, allowing for GPIb-dependent platelet adhesion at high-shear conditions *in vitro* (17). However, TSP-1 also has been shown to affect thrombosis by protecting (sub)endothelial VWF from cleavage by ADAMTS13 (47), a mechanism unrelated to its role as a ligand for GPIbα. P-selectin, an adhesion receptor found on activated platelets and endothelial cells, is also a ligand for GPIbα (11). Vascular injury by $FeCl_3$, however, leads to rapid endothelial denudation (5), making it unlikely that the P-selectin–GPIbα interaction plays a role in platelet adhesion to the vessel wall, even in the absence of VWF. P-selectin, on the other hand, could play a role in platelet adhesion to thrombi, and defects in thrombus growth were shown in the presence of anti-P-selectin antibodies (48). Further studies in mice with combined mutations in VWF and TSP-1 or VWF and P-selectin are needed to clarify this question.

In summary, we have shown that GPIbα is absolutely required for platelet adhesion/thrombus formation under high-shear conditions as found in mesenteric arterioles. Because platelets and platelet adhesion also contribute to various other pathophysiological processes including inflammation, heart disease, and angiogenesis (49–51), inhibitors of GPIbα that interfere with platelet adhesion might become powerful drugs in the future.

Materials and Methods

Animals. IL4Rα/GPIbα transgenic (22), β3-deficient (27), and the respective control mice were bred and housed in our facilities. Experimental procedures were approved by the Animal Care and Use Committee of the CBR Institute for Biomedical Research.

Reagents and Antibodies. The following reagents were used: Lovenox (enoxaparin sodium) (Aventis Pharmaceuticals Products, Sanofi-Aventis, Bridgewater, NJ), heparin-coated microcapillaries (VWR Scientific, West Chester, PA), collagen reagent Horm (NYCOMED, Munich, Germany), BSA (Chrono-log Corp., Haverton, PA), prostacyclin (PGI_2), thromboxane A_2 mimetic U46619, human thrombin, ADP, and ferric chloride ($FeCl_3$) (all from Sigma, St. Louis, MO), *O*-sialoglycoprotein endopeptidase (Cedarlane, Hornby, ON, Canada), Fluo-3, calcein AM (calcein-green), and calcein red-orange AM (all from Molecular Probes, Eugene, OR). Monoclonal antibodies against human IL4Rα, mouse CD62P, and mouse α2β1 were from BD Pharmingen, San Diego, CA. Antibodies against mouse receptors αIIbβ3, GPVI, GPV, GPIX, and GPIbα were from emfret Analytics (Wuerzburg, Germany).

Flow Cytometry. ***Expression of adhesion receptors on resting platelets.*** Mice were bled from the retro-orbital plexus under isoflurane anesthesia (IsoFlo; Abbott Laboratories, Abbott Park, IL). Blood (7 volumes) was collected into a tube containing 30 units/ml heparin in PBS (pH 7.4, 3 volumes) and diluted with modified Tyrode-Hepes buffer (137 mmol/liter NaCl/0.3 mmol/liter Na_2HPO_4/2 mmol/liter KCl/12 mmol/liter $NaHCO_3$/5 mmol/liter Hepes/5 mmol/liter glucose, pH 7.3) containing 0.35% BSA. Platelets (2×10^6) in diluted whole blood were incubated with saturating amounts of the indicated FITC-labeled antibodies for 20 min at room temperature (RT) and analyzed immediately on a FACScalibur (Becton Dickinson, Rockville, MD).

Expression of activated αIIbβ3 on stimulated platelets. Platelet-rich plasma was obtained from heparinized whole blood by centrifugation at $300 \times g$ for 10 min. Platelet-rich plasma was centrifuged at $1{,}000 \times g$ in the presence of PGI_2 (0.1 μg/ml) for 7 min at RT. After two washing steps, pelleted platelets were resuspended in modified Tyrode-Hepes buffer containing 0.35% BSA and 1 mM $CaCl_2$. Platelets (2×10^6) were activated with the indicated concentrations of thrombin or ADP (in the presence of 5 μM U46619) for 10 min at RT, stained with PE-labeled antibodies against activated αIIbβ3 (JON/A-PE (52), 10 min at RT), and immediately analyzed. Platelets were gated by forward scatter/side scatter characteristics.

Calcium flux. Washed platelets were incubated for 15 min with 5 μM Fluo-3, stimulated with the indicated agonist concentrations, and immediately analyzed. Platelets were gated by forward scatter/side scatter characteristics.

Treatment of Platelets with *O*-Sialoglycoprotein Endopetidase. The N-terminal domain of GPIbα was removed from platelets as described in ref. 23. Briefly, washed platelets (2×10^9/ml) were resuspended in modified Tyrode's buffer containing 1 mM $CaCl_2$ and incubated at 37°C for 30 min with 250 μg/ml *O*-sialoglycoprotein endopeptidase. Aliquots of the platelet suspensions were analyzed by flow cytometry to detect alterations in GPIbα expression.

Aggregometry. Light transmission was measured by using platelet-rich plasma (ADP, collagen, U46619) or washed platelets (thrombin) adjusted to 3×10^8 platelets per ml with modified Tyrode's buffer containing 1 mM $CaCl_2$. Agonists were added at the indicated concentrations and transmission was recorded over 12 min on a Chrono-log 4-channel optical aggregation system (Chrono-log Corp.).

***In Vivo* Thrombosis Model.** Platelets were labeled for 10 min with calcein-green (5 μg/ml) or calcein-red/orange (2.5 μg/ml) and infused into 3- to 5-week-old anesthetized male mice. The mesentery was exposed through a midline abdominal incision. Vessels with a shear rate of 1,000–1,400 sec^{-1} were selected by using an Optical Doppler Velocimeter (Texas A&M University System Health Science Center, Cardiovascular Research Institute, College Station, TX). Arterioles were visualized with a Zeiss Axiovert 135-inverted microscope (Zeiss, Oberkochen, Germany) and adhesion of fluorescently labeled platelets was monitored in black and white with a silicon-intensified tube camera (C2400-08; Hamamatsu, Hamamatsu City, Japan) connected to an S-VHS video recorder (AG-6730; Panasonic, Matsushita Electric, Japan). Vessel injury was generated by using a filter paper (1×4 mm) soaked with a 10% $FeCl_3$ solution and placed over the vessel for 5 min. The paper then was removed, and the vessel was covered with saline at 37°C. Vessels were monitored for 40 min after $FeCl_3$ treatment or until cessation of blood flow lasted >10 s (occlusion).

To determine platelet tethering to injured vessels, 10 μl of $FeCl_3$ was superfused over mesenteric arterioles, and the number of fluorescently labeled platelets tethering to vessel walls was counted over a 400-μm stretch of vessel.

For studies on the adhesion of activated platelets, calcein-labeled cells were treated with human thrombin (0.2 unit/ml) for 5 min at 37°C in presence of 0.5 mM EDTA to avoid aggregation. Hirudin (1 unit/ml) was added to stop the reaction. The activation was confirmed by surface expression of P-selectin. Platelets were infused intravenously so that they represented ≈2% of platelets in circulation.

In all experiments comparing the adhesion of two sets of platelets, the labeling with calcein-green and calcein-red/orange was alternated between the two sets of platelets.

Statistical Analysis. Data are presented as mean ± SEM. The differences in surface receptor expression, platelet adhesion, and thrombus formation were analyzed by unpaired, two-tailed Student's *t* test. The differences in platelet incorporation into growing thrombi were analyzed by Wilcoxon Signed Rank test. *P* values <0.05 were regarded as statistically significant.

Supporting Information. Movies show platelet adhesion/thrombus formation in response to arteriolar injury in WT (Movie 1, which is published as supporting information on the PNAS web site) and IL4Rα/GPIbα transgenic (Movie 2, which is published as supporting information on the PNAS web site) mice. The experimental detail is described in Fig. 2 legend.

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